Moderate folate depletion modulates the expression of selected genes involved in cell cycle, intracellular signaling and folate uptake in human colonic epithelial cell lines

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Abstract

Folate deficiency may affect gene expression by disrupting DNA methylation patterns or by inducing base substitution, DNA breaks, gene deletions and gene amplification. Changes in expression may explain the inverse relationship observed between folate status and risk of colorectal cancer. Three cell lines derived from the normal human colon, HCEC, NCM356 and NCM460, were grown for 32–34 days in media containing 25, 50, 75 or 150 nM folic acid, and the expression of genes involved in cell-cycle checkpoints, intracellular signaling, folate uptake and cell adhesion and migration was determined. Expression of Folate Receptor 1 was increased with decreasing media folate in all cell lines, as was p53, p21, p16 and β-catenin. With decreasing folate, the expression of both E-cadherin and SMAD-4 was decreased in NCM356. APC was elevated in NCM356 but unchanged in the other lines. No changes in global methylation were detected. A significant increase in p53 exon 7–8 strand breaks was observed with decreasing folate in NCM460 cells. The changes observed are consistent with DNA damage-induced activation of cell-cycle checkpoints and cellular adaptation to folate depletion. Folate-depletion-induced changes in the *Wnt*/APC pathway as well as in genes involved in cell adhesion, migration and invasion may underlie observed relationships between folate status and cancer risk.

1. Introduction

Human population studies show that a high dietary folate intake is associated with a diminished risk of colorectal cancer [1–3] and, possibly, of cancers of the breast [4–6], uterine cervix [7–9], lung [10,11], esophagus and pancreas [12] as well. The inverse association between folate status and the risk for carcinogenesis is thought to be explained by the role of folate coenzymes in providing one-carbon moieties for nucleotide and *S*-adenosylmethionine (via methionine) synthesis [13].

In cell culture, folate depletion increases the intracellular dUMP:dTMP ratio by up to 10-fold [14,15]. In vitro [15–18] and in vivo [19–21] studies reveal that this nucleotide imbalance promotes the misincorporation of uracil into DNA and the subsequent formation of double-stranded DNA breaks (DSB) through the excision of closely spaced uracil residues on opposing strands during repair [22]. Low dietary folate intake promotes genomic strand breakage in the liver of rats [21,23,24] and micropigs [25]. In addition, folate-depletion-induced DNA breakage has been observed in primary human lymphocytes [17,26] and rodent cell lines [15,27] in vitro. Folate-deficiency-induced chromosome breakage is relevant because the accumulation of chromosome aberrations, such as double-stranded breaks, is an established risk factor for the development of cancer [28].

In addition to disturbing nucleotide metabolism, folate depletion is reported to cause genomic hypomethylation in humans [19] and in cell culture [29] and p53 gene-specific hypomethylation in rats [23,24]. Although a depletion of the universal methyl donor *S*-adenosylmethionine is observed during folate depletion, the accumulation of its precursor *S*-adenosylhomocysteine, an inhibitor of DNA methyltransferases, appears to be a stronger predictor of hypomethylation [30]. Interestingly, under folate-depleted conditions, specific sequences may also become hypermethylated, as is the case with the H-cadherin gene [31]. DNA methylation is an important mechanism of gene silencing because the majority of tissue-specific genes exhibit a strong correlation between hypomethylation of the promoter region and gene expression [32].

These deleterious genetic and epigenetic changes likely elicit a change in cellular gene expression profiles, whether as a response to, or as a consequence of, a modified template. Several groups, including our own, have begun characterizing the effect of low folate status on the transcriptome using both rodent and cell culture models.

Previous studies have shown that Folate Receptor 1 (FOLR1) [33–36] is up-regulated due to folate depletion in vitro, while it is the reduced folate carrier (RFC) that is up-regulated in the rat intestine [37,38], with the FOLR1 remaining undetectable [38]. Increased FOLR1 in vitro and the increased RFC in vivo reflect the abundance of available forms in the particular model and the affinity of each protein for them. FOLR1 has a higher affinity than RFC for folic acid (pteroylglutamic acid), the primary folate form in culture media. Conversely, the predominant folate form in plasma is 5-methyltetrahydrofolate, a form for which RFC has a greater affinity [39]. Nevertheless, these changes appear to be an adaptive response as cells attempt to take up more folate from their surroundings.

In addition to these adaptive changes, an array of interesting genes seem to be responsive to folate status. Among these are genes involved in implementing the cell-cycle checkpoints and DNA repair as well as genes involved in cell adhesion, motility and invasion. These two categories of genes are of clear relevance to carcinogenesis as they both include tumor suppressors and genes up-regulated in tumors.

To date, the reported response of cell-cycle checkpoint genes such as *p21* and *p53* to folate status has been varied. Folate depletion resulted in reduced p53 expression in the rat colon [40,41], which was associated with increased p53 strand breaks [40]. Similarly, p53 was decreased in HCT116, HT29 and LS513 but increased in Caco-2 colon cancer cells [42]. We recently observed an increase in p53 transcript and protein, despite substantial elevations in strand breaks, in primary human lymphocytes [43]. Immediately downstream of p53, p21 was also studied in colon cancer cells and was reported to be elevated in HCT116 and Caco-2 cells but down-regulated in HT29 and LS513 cells [42].

In the second category, Jhaveri et al. [31] noted the hypermethylation-related down-regulation of H-cadherin, a cell adhesion molecule, in folate-depleted KB cells. Similarly, we observed a reduction in the protocadherin-4 adhesion molecule in the colon of folate-depleted rats. This was accompanied by a concomitant reduction in attachment factors; integrin α5, glypican 1, nidogen, involucrin and proteases; MMP16, TIMP-2, metalloendopeptidase, metallothionein and subtilisin and an elevation of urokinase and chymase [44]. Others report an elevation of metallothionein in Chinese hamster lung fibroblasts selected for growth in low folate [45]. The elevation of urokinase, as well as its receptor, under folate depletion, was observed by others in HCT116 but not in Caco-2 cells [42].

Emerging data also indicate that the APC/*Wnt* pathway, which is commonly disrupted in colon carcinogenesis [46], is sensitive to folate status. Novakovic et al. [42] report an elevation of β-catenin transcript in three of four colon cancer cell lines. Furthermore, we have recently observed that multiple but mild B-vitamin depletion (folate, B_2, B_6 and B_{12}) induced an elevation of β-catenin protein and a decrease in APC gene expression, which was associated with elevated DNA breaks in the APC mutation cluster region (Liu et al., in preparation).

In light of these findings, we sought to further characterize the impact of folate status on the expression of genes involved in cell-cycle checkpoints, cell adhesion and migration and in the APC/*Wnt* pathway. Furthermore, since it remains unclear in the instance of one gene (*p53*), we sought to determine whether the induction of folate-dependent strand breaks in the coding region of the gene is related to diminished expression. Such studies may help us understand the mechanisms underlying the observed inverse relationship between cancer risk and folate status. Folate status has previously been observed to have a differential effect on tumorigenesis in animals, with supplementation being protective if started before the appearance of precancerous lesions while promoting tumor growth after initiation has already occurred [47,48]. For this reason, we have chosen to perform our experiments in cells derived from the normal colon, which, to the best of our knowledge, are among the most 'normal' colonic cells currently available. We cultured HCEC, NCM460 and NCM356 cell in media containing 25–150 nM folic acid for 30 days and studied the expression of genes involved in cell-cycle checkpoints (*p16*, *p21*, *p53*), adhesion and migration (*urokinase*, *E-cadherin*), the *Wnt* pathway (*β-catenin*, *APC*), folate uptake (*RFC*, *folate receptor*) and signaling (*SMAD-4*).

2. Methods

Three colonic epithelial cell lines derived from the normal adult human colon were cultured for 30–32 days

in media containing varying concentrations of folic acid. Human colonic epithelial cells (HCECs) were a gift from Dr. A. Pfeifer (Nestle, Lausanne, Switzerland). HCECs were obtained from a scratch biopsy of a 69-year-old female, are SV-40 transformed and do not express cytokeratin 19. NCM356 and NCM460 cells were obtained from INCELL (San Antonio, TX). NCM cells are not transfected with any exogenous genetic material and do express cytokeratin 19 (data not shown). NCM356 and NCM460 are derived from the normal mucosa of 65- and 68-year-old males, respectively. Cells were grown in T75 flasks in 10 ml of A52 media supplemented with 30 μg/ml bovine pituitary extract, 100 nM retinoic acid (Biofluids, Rockville, MD, now Invitrogen, Carlsbad, CA), 8 μg/ml vitamin C, 2 mM L-glutamine and 1 nM dexamethasone. A52 was custom made without folic acid, which was added at concentrations of 25, 50, 75 and 150 nM before use. HCECs require the precoating of flasks with Matrigel extracellular matrix solution (BD Biosciences, San Jose, CA).

DNA was extracted from cells using phenol:chloroform via standard procedures and quantified using Pico green dye (Invitrogen). Total cellular RNA was isolated from cells using Trizol reagent, and cDNA was synthesized using Superscript II reverse transcriptase (both from Invitrogen).

The relative expression of selected transcripts was determined using Taqman gene expression assays and PCR mastermix with an ABI7300 thermal cycler according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Gene expression was normalized to that of GAPDH (ΔCt) and is reported as relative to the control group by using the following equation: relative expression$=2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct is the ΔCt of the test group (25, 50 and 75 nM folate) minus the ΔCt of the control group (150 nM).

The integrity of exons 7–8 of the p53 tumor suppressor gene was studied using a PCR-based assay [43] whereby damage such as double-stranded DNA breaks inhibits the progression of Taq polymerase and thereby decreases amplification rate. Briefly, 100 ng of DNA was subjected to PCR for p53ex7–8 and GAPDH. An increase in ΔCt ($Ct_{p53}-Ct_{GAPDH}$) indicates a reduction in amplifiable template or increase in DNA breakage.

Genomic DNA methylation was determined in 1 μg DNA by enzymatic hydrolysis of DNA followed by LC-MS using the method of Friso et al. [49].

Data for each folic acid concentration were compared using one-way ANOVA with Tukey's post hoc tests, and significance was accepted when $P\leq.05$. In addition, linear regression was used to test the relationship between ΔCt and medium folate concentrations. Data are expressed as mean±S.E.M. All calculations were performed using SPSS Systat v10 (Point Richmond, CA). For gene expression, ΔCt values were analyzed; however, relative expression values are reported here for brevity and clarity.

3. Results

Decreasing media folate concentration caused an incremental reduction in cell growth for HCEC and NCM356 cells, while in NCM460, cell growth was similar for 50–150 nM and reduced only at 25 nM (Fig. 1).
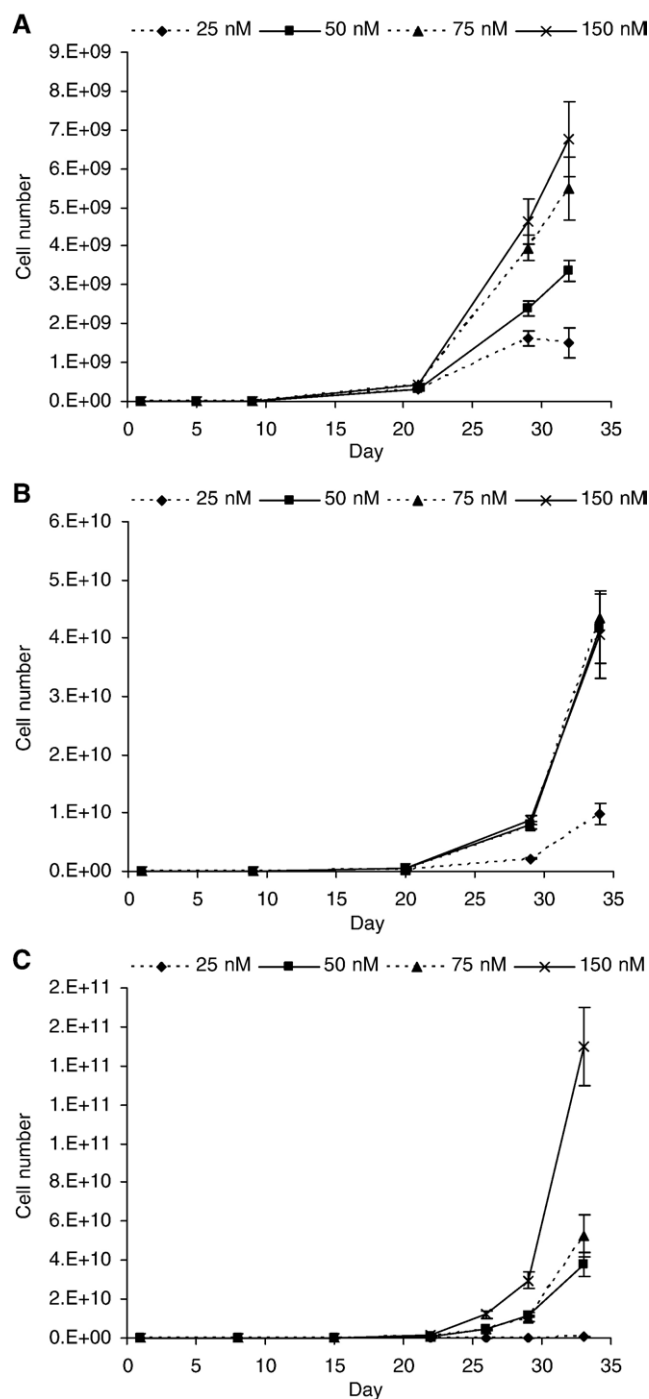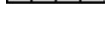

Fig. 1. Growth curves for colonic epithelial cells cultured with varying concentrations of folic acid. (A) HCEC, (B) NCM460, (C) NCM356. Data are expressed as mean±S.E.M.

Table 1
Effect of media folic acid concentration on relative gene expression in colon epithelial cell lines

Gene	Cell line	Media folic acid (nM)				ANOVA P	Trend
		25	50	75	150		
Reduced Folate Carrier	HCEC	1.78[a]	0.83	0.56[a]	1.00	.002	
SLC19A1	NCM460	1.03	0.76	0.60	1.00	.106	
NM_003056.2	NCM356	1.11	0.49	0.56	1.00	.061	
Folate Receptor 1	HCEC	5.17	4.55	2.57	1.00	.101[b]	
FOLR1	NCM460	9.59[a]	0.93	1.11	1.00	<.001	
NM_016724.1	NCM356	132.51[a]	49.92[a]	15.82[a]	1.00	<.001[b]	
Adenamatosis Polyposis Coli	HCEC	1.21	1.50	1.06	1.00	.660	
APC	NCM460	1.52	1.01	1.05	1.00	.310	
NM_000038.3	NCM356	1.34[a]	1.23	1.37[a]	1.00	.018[b]	
β-Catenin	HCEC	1.30	1.48	1.14	1.00	.150[b]	
CTNNB1	NCM460	1.46[a]	0.89	0.89	1.00	.003	
NM_001904.2	NCM356	1.97[a]	2.89[a]	2.38[a]	1.00	<.001[b]	
E-Cadherin	HCEC	1.88	1.62	0.98	1.00	.550	
CDH1	NCM460	1.43	1.57[a]	1.42	1.00	.048[b]	
NM_004360.2	NCM356	0.25[a]	0.28[a]	0.45[a]	1.00	<.001[b]	
Urokinase	HCEC	3.23[a]	2.79[a]	1.88[a]	1.00	.001[b]	
PLAU	NCM460	2.29[a]	1.26	1.00	1.00	<.001[b]	
NM_002658.2	NCM356	1.16	0.74	0.85	1.00	.062	
p53	HCEC	2.06[a]	1.87[a]	1.73[a]	1.00	.003[b]	
TP53	NCM460	1.23[a]	1.20[a]	1.16[a]	1.00	.007[b]	
NM_000546.2	NCM356	5.20[a]	4.23[a]	3.03[a]	1.00	<.001[b]	
p21	HCEC	3.28[a]	1.70	1.28	1.00	.004[b]	
CDKN1A	NCM460	1.34	1.27	0.90	1.00	.117	
NM_078467.1	NCM356	7.06[a]	4.92[a]	3.23[a]	1.00	<.001[b]	
p16	HCEC	1.66	1.15	0.89	1.00	.051	
CDKN2A	NCM460	1.37	1.14	1.11	1.00	.073[b]	
NM_058195.2	NCM356	3.20[a]	2.18[a]	1.56[a]	1.00	<.001[b]	
SMAD-4	HCEC	2.19[a]	2.17[a]	1.54	1.00	.013[b]	
SMAD-4	NCM460	1.12	1.24	1.14	1.00	.070	
NM_005359.3	NCM356	0.15[a]	0.11[a]	0.34[a]	1.00	<.001[b]	

Relative expression=$2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct = \Delta Ct_{test\ group} - \Delta Ct_{control\ group}$. $\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$. Statistics were performed on ΔCt values. [a]$P \le .05$ vs. 150 nM (Tukey). [b]P trend (regression)<.05. Gene symbols and accession numbers are listed below the gene name. Trends for gene expression change are illustrated with black graphs indicating significant trends.

Gene expression data are reported in Table 1 as relative expression values.

Decreasing media folate caused a substantial elevation in the expression of FOLR1 in all three cell lines, especially in NCM356, which displayed a 132-fold elevation in FOLR1 at 25 nM, as compared with 150 nM. Interestingly, there is a tendency for the RFC to be slightly suppressed at the middle folate deficiencies and then normalize or slightly elevated at 25 nM, a trend that is significant in HCEC.

Table 2
Effect of media folic acid concentration on genomic DNA methylation in colon epithelial cell lines

Cell line	Media folic acid (nM)				ANOVA P
	25	50	75	150	
HCEC	3.14±0.29	2.53±0.19	2.95±0.67	1.94±0.06	.20
NCM460	3.81±0.12	3.82±0.19	3.76±0.32	4.19±0.29	.58
NCM356	3.04±0.15	3.01±0.28	2.27±0.23	2.81±0.13	.09

DNA methylation is reported as nanograms of 5-methylcytosine per microgram of DNA. Data are expressed as mean±S.E.M.

Decreasing media folate caused an incremental up-regulation of the DNA damage and cell-cycle-checkpoint-related genes *p53*, *p16* and *p21*. This response is especially robust for p53, which was significantly up-regulated in all cell lines for even the mildest deficiency concentration of 75 nM, a concentration that could support maximal cell proliferation in NCM460. The trend for up-regulation of p21 is significant in HCEC and NCM356 but equivocal in NCM460. Similarly, p16 is significantly up-regulated in the folate-deficient NCM cell lines, but the response in HCEC is marginal.

Both APC and β-catenin are incrementally up-regulated during folate depletion in NCM356 cells ($P<.05$), a trend that does not reach significance in the other cell lines. E-cadherin, another member of the *Wnt* pathway, is down-regulated in NCM356 cells ($P<.05$) but marginally elevated in NCM460 and HCEC. Possibly related to these changes is SMAD-4, an inducer of E-cadherin, which is also significantly down-regulated in NCM356 but marginally elevated in NCM460 and HCEC under folate-deficient conditions.

Urokinase expression was elevated at the three deficiency folate concentrations in HCEC and at 25 nM in NCM460 but was unchanged in NCM356.

Genomic DNA methylation was not affected by folate status in any of the cell lines ($P>.05$, Table 2).

Media folate concentration did not affect p53 exon 7–8 strand breakage in HCEC and NCM356 but caused a stepwise increase in strand breaks in NCM460 cells (Fig. 2). A 1.96 cycle difference in ΔCt was observed between the 25- and the 150-nM folate groups. Based on our experiments with restriction-digested DNA [43], we estimate this to represent an approximately 23.1% reduction in intact p53 exon 7–8 DNA template.
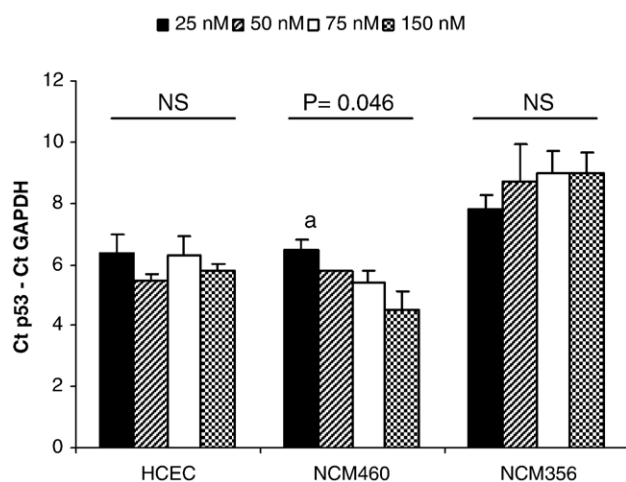

Fig. 2. Effect of media folic acid concentration on p53 exon 7–8 integrity in colon epithelial cell lines. Increased ΔCt indicates increased strand breakage within p53 exons 7–8. Data are expressed as mean±S.E.M. [a]$P<.05$ vs. 150 nM. NCM460 *P* trend (regression)=.004.

4. Discussion

We studied the impact of folate depletion on the expression of select genes involved in folate uptake, cell adhesion and migration and cell-cycle checkpoint regulation, all of which are relevant to carcinogenesis.

A strong up-regulation of FOLR1, but not RFC, due to folate depletion was an anticipated observation due to the high affinity of FOLR1 but not RFC for folic acid [39]. This adaptive mechanism has previously been shown to be mediated by an increase in transcription rate and transcript half-life [33,36]. It is noteworthy that in NCM460 cells, FOLR1 expression and cell proliferation are similar for the upper three folate concentrations and then significantly increase and decrease at 25 nM folate, respectively. Although comparisons of relative expression cannot be made between the cell lines, it is likely that NCM460 cells have a much higher basal FOLR1 expression than HCEC and NCM356 cells, which is sufficient to support maximal cell growth down to folate concentrations of 50 nM.

Folate depletion induced the up-regulation of *p16*, *p21* and *p53* tumor suppressor genes, which are involved in DNA damage signaling, inhibition of cell-cycle progression through checkpoints and apoptosis. It is likely that folate depletion caused uracil incorporation and DNA breaks [26], thereby activating the DNA repair protein ATM. ATM is known to activate p53, via Chk2, which, in turn, stimulates p21 transcription. p21 arrests the cell cycle by inhibiting CDK2/cyclin E while p16 inhibits CDK4,6/cyclin D. Because folate-depletion-induced damage is expected to occur during S-phase when uracil is incorporated into newly synthesized DNA, activation of the CDK inhibitors p16 and p21 would block the transition of cells out of this phase of the cell cycle. In support of this, the accumulation of cells in S-phase has been observed in folate-depleted lymphocytes [50]. It should be noted that HCECs were immortalized using the SV40-T antigen, which is known to bind and inhibit p53 protein function [51]. Nevertheless, the folate-depletion-induced increase in p53 transcript in HCECs was of a similar magnitude to the non-SV40 immortalized NCM cells. One might infer that since p21 transcript is also similarly elevated across the three cell lines, the SV40-T antigen does not significantly impair the ability of p53 to induce downstream genes such as p21 transcription in HCECs in this system.

We have previously observed a folate-depletion-induced up-regulation of p53 transcript and protein in primary human lymphocytes despite 26% and 40% reductions in amplifiable template from exons 7–8 and 5–8 of the gene, respectively [43]. This demonstrates that the induction of strand breaks is not invariably accompanied by a reduction in the corresponding transcript. In the current study, NCM460 showed a similar pattern: the degree of p53 exon 7–8 template loss in depleted compared to replete

cells (23%) was accompanied by a significant increase in steady-state p53 mRNA. Interestingly, no loss of amplifiable template was observed in either HCEC or NCM356 cells (Fig. 2). As this assay is a measure of intact gene template, the copy number of the target gene is a central determinant of the result and, as such, might only be reliable in cells with a diploid karyotype. We have previously determined the HCECs to be almost tetraploid (72.13±1.03 chromosomes) and NCM460 to be almost diploid (56.47±0.63 chromosomes) but have not determined the karyotype of NCM356.

Folate depletion has been shown to induce p53 stand breaks with a concomitant reduction in steady-state p53 mRNA in the rat colon [40]. In this tissue, unlike our cell culture models, the severity of p53 gene loss or damage may have overwhelmed cellular mechanisms aimed at maintaining elevated p53 during genotoxic stress, thereby increasing the chance that cells with deleterious genetic or epigenetic aberrations continue dividing, a procarcinogenic state.

In agreement with our rat studies [44], folate depletion induced the up-regulation of urokinase in HCEC and NCM460 cells. Others observed an up-regulation in HCT116 but not in Caco-2 cells [42]. Urokinase is involved in ECM remodeling and cell migration and, when elevated in primary tumors, is a predictor of metastasis and poor prognosis for breast cancer patients [52]. The observations that cadherins, urokinase and various other attachment factors and ECM proteases are affected by folate status [31,53] suggest that cell adhesion, migration and ECM remodeling, important processes in progression of established lesions, may be a novel mechanism, in addition to genetic/epigenetic instability, by which folate status modulates our risk for cancer.

It is not clear how or why folate status affects urokinase transcript levels; however, a complex interplay between multiple genes seems to be involved. Activation of urokinase has been shown to be dependent on E-cadherin, at least at the protein level [54]. In our system, the two cell lines that have a folate-depletion-induced elevation of E-cadherin (HCEC and NCM460) are also the ones that display an increase in urokinase. Regulation of urokinase has also been attributed to SMAD-4 [55] and β-catenin [56]. Furthermore, SMAD-4 is an inducer of E-cadherin transcription [57], and in our system, the changes in these two genes are paralleled, with a trend for increase in HCEC and NCM460 and decrease in NCM356 during folate depletion. A detailed survey of protein levels is clearly required to further decipher this scheme.

Our data, along with those of others, indicate that the *Wnt* pathway is clearly affected by cellular folate status. In addition to the abovementioned changes in β-catenin and E-cadherin, which has an additional role in sequestering β-catenin, APC is also affected by folate status, at least in NCM356 cells. Similarly, we have also observed a reduction in APC transcript in the mouse colon due to multiple B-vitamin deficiency (Liu et al., submitted for publication).

Wnt signaling pathway members APC and β-catenin are mutated or lost in the majority of the sporadic colorectal cancer cases, resulting in activation of the pathway, which has a diverse array of transcriptional targets.

We did not observe any alteration in genomic DNA methylation due to folate depletion in our system (Table 2). This may be due to the fact that DMEM, which is the base for A52 media, contains a high concentration of methionine (30 mg/L or 200 μM), thereby negating the cellular need for folate to recycle homocysteine to methionine. In future experiments, titrating the amount of methionine in the media down to the minimal level that supports normal proliferation may precipitate an effect of folate depletion on global DNA methylation and thereby amplify the gene expression response.

Emerging data seem to indicate that folate status affects several cancer-related pathways including the p53 pathway (p53 and p21), the Rb pathway (p16) and the APC/*Wnt* pathway (CTNN1 and CHD1), as well as pathways involved in cell adhesion (CDH1) and cell migration and invasion (PLAU1). Folate-induced changes in these pathways may underlie the observed associations, whether positive or negative, between folate intake and cancer incidence.

Acknowledgments

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